DEVELOPMENTAL FACTORS AFFECTING REGENERATION IN THE CENTRAL NERVOUS SYSTEM: EARLY BUT NOT LATE FORMED MITRAL CELLS REINNERVATE OLFACTORY CORTEX AFTER NEONATAL TRACT SECTION

BY

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You can live to be 100 and still learn something about melons.
-Anonymous

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Chairman: Christiana M. Leonard
Major Department: Department of Neuroscience

The olfactory system of the neonatal golden hamster has a known capacity for behavioral and neuroanatomical plasticity. If the lateral olfactory tract (LOT) is transected in the first postnatal week, axons grow through the cut and reinnervate the terminal regions. Functional recovery occurs only when the terminal regions are reinnervated. These experiments examined the possibility of a specific anatomical principle regulating axonal regrowth and reinnervation after early lesions. The original hypothesis was that the reinnervation arises from continued growth of newly-formed axons which were not severed by the lesion.

The first experiment determined the time of cell formation of neurons in the hamster olfactory bulb. Mitral cells are formed on gestational days 11 and 12 (E11 and E12), and tufted cells on E11 to E14. There is an outward progression of the positions within the external plexiform layer (EPL) of cells formed from E10 to E14.

The second experiment involved the combination of ^{3}H-thymidine labeling for time of cell formation with the retrograde transport of horseradish peroxidase (HRP). Axons of early-formed cells reach the olfactory cortex before those of later-formed cells. This is believed to be the first demonstration of the correlation of times of cell formation and axonal projection in individual cells.

The third experiment examined the possibility that axons which grow through an early LOT transection are new axons which had not yet reached the level of the cut. Animals were given ^{3}H-thymidine on E11 or E13, and a transection of the LOT on day 3. After a recovery period sufficient to allow axonal regrowth, HRP was placed in the olfactory projection regions caudal to the prior LOT section. The original hypothesis was not supported. Cells which are formed early, and send out their axons early, reinnervate the olfactory cortex, while late-formed cells do not. Early LOT section decreases the total number of mitral cells, and affects the positions of tufted cells.

Evidence was found for a mildly toxic effect of ^{3}H-thymidine injections. The positions of cells destined for the EPL were specifically affected by the incorporation of ^{3}H-thymidine on E13.

CHAPTER I
INTRODUCTION

In the past twenty years, the use of ^{3}H-thymidine autoradiographic techniques has provided much information about the development of the central nervous system (CNS). This method has revealed the time and place of origin of CNS cells, the cell lines from which neurons and glia are derived, and the paths which these cells follow in their migrations from site of origin to final position. The technique is limited, however, in that, by itself, it cannot give information about the connections of cells or the development of these connections, or how these processes are related to the functional development of the brain.

This study combines ^{3}H-thymidine autoradiography with the retrograde transport of horseradish peroxidase (HRP) and neonatal lesions in an attempt to directly investigate possible correlations in the time of cell formation and the development of their axonal projections. The combination of ^{3}H-thymidine and HRP has been used previously to examine whether cells within a given region with different birthdates ultimately project to different, or the same regions (Nowakowski et al., 1975). They found that mouse hippocampal neurons formed on gestational days 13 and 14 have similar projections to the contralateral hippocampus. The study we are now reporting looks at whether individual cells within a region, which are known to ultimately project to similar areas, do so in a sequence which correlates with their time of origin.

The olfactory bulb, and its projection to the olfactory cortical regions of the basal forebrain, is a convenient system in which to examine this question. The olfactory bulb sits in the front of the brain, is well laminated, and has a discrete, easily identifiable population of cells which project centrally. The axons of the mitral and tufted cells leave the bulb in a distinct bundle, the lateral olfactory tract (LOT), which travels on the surface of the brain to its projection areas in the basal forebrain. Along the course of the LOT, many collaterals branch off and project into the anterior olfactory nucleus, olfactory tubercle, piriform cortex, and other basal forebrain regions (Devor, 1976a). The bulb, LOT, and olfactory cortex are all easily accessible with little disruption of the rest of the brain.

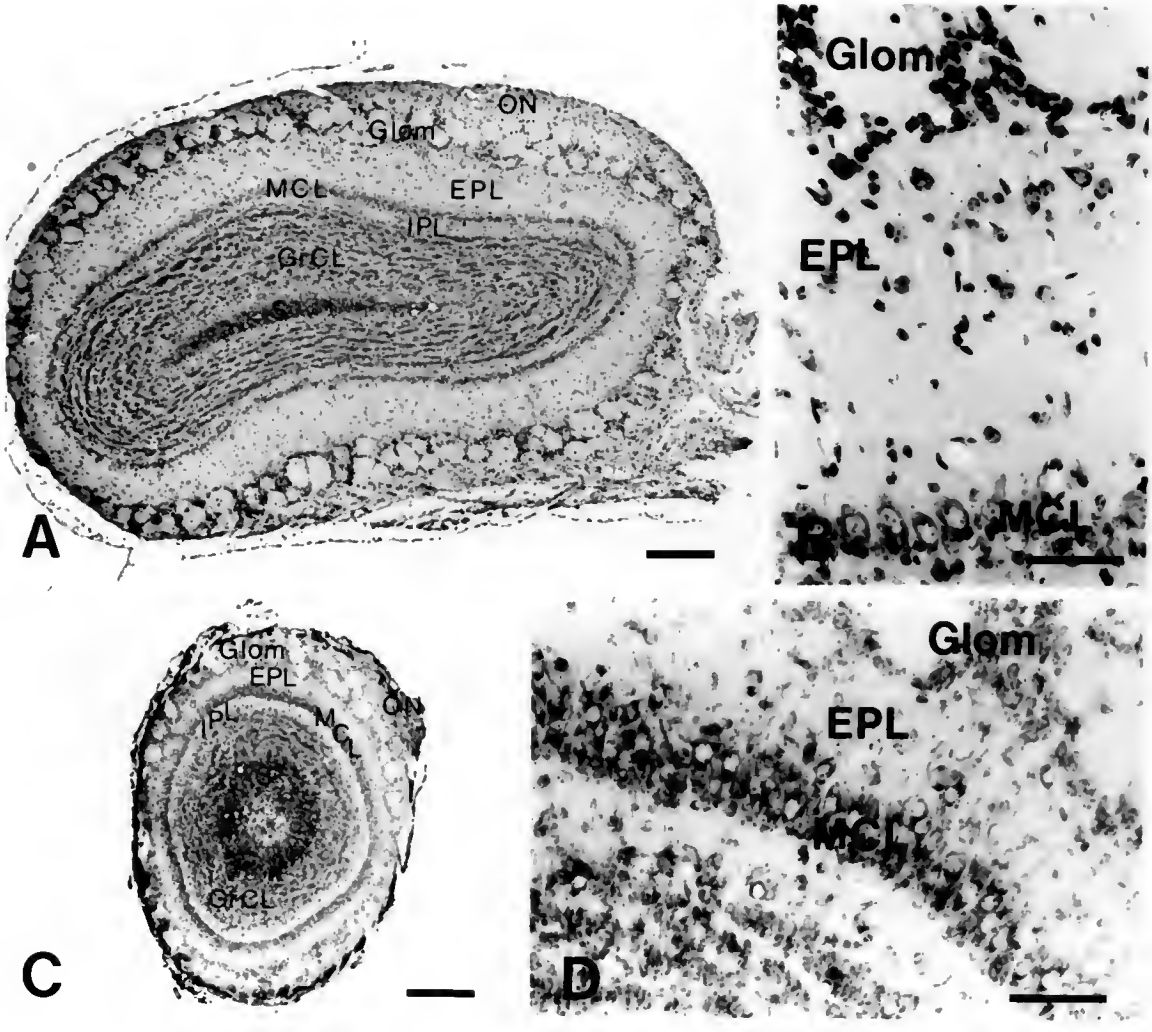
The rodent olfactory system is behaviorally functional in the neonate (Devor and Schneider, 1974; Cornwell, 1975; Blass et al., 1977; Rudy and Cheatle, 1977; Crandall and Leonard, 1979), but undergoes both functional and anatomical changes during the first few weeks of postnatal life (see, for example: Devor and Schneider, 1974; Leonard, 1975; 1978; Singh and Nathaniel, 1977; Schwob and Price, 1978; Crandall and Leonard, 1979). The time of origin of olfactory bulb cells, their pattern of migration, and their differentiation have been extensively studied in the mouse by Hinds (1968a,b; 1972; Hinds and Ruffet, 1973). The postnatal establishment and expansion of the LOT projections in rat and hamster have been described using degeneration (Leonard, 1975; Singh, 1977), autoradiographic (Schwob and Price, 1978), and electron microscopic methods (Westrum, 1975). The LOT projection also has a capacity for functional and anatomical plasticity during the neonatal period (Devor, 1975; 1976b; Small, 1977).

Devor (1975) showed that recovery of male hamster mating behavior, which is olfactory-dependent, occurs only when the terminal fields caudal to the cut are reinnervated after an early LOT section. Following early LOT sections, pups which showed functional recovery on a thermal behavior test were found to have reinnervation of the piriform cortex and the lateral part of the olfactory tubercle (Small, 1977).

Much of the behavioral work on olfactory development has been done with the golden hamster, including Devor's work showing behavioral and neuroanatomical plasticity. For this reason, we chose to use the hamster in our studies of the LOT projections. The hamster offered the additional advantage of a very short gestational period, so that it was possible to make postnatal, rather than fetal manipulation of the system.

Before detailing the experimental rationales, a description of the anatomy of the olfactory bulb and its projection neurons is necessary. The rodent olfactory bulb consists of the following layers (Figure 1): 1) the superficial olfactory nerve layer, 2) the glomerular layer, where the olfactory nerve axons synapse with olfactory bulb dendrites, 3) the external plexiform layer (EPL), 4) the mitral cell body layer (MCL), 5) a cell-poor region of neuropil--the internal plexiform layer, 6) the granule cell layer, with densely packed granule cell perikarya, and 7) the ventricular/subventricular layer (Ramon y Cajal, 1911). The subventricular zone persists in the adult olfactory bulb (Hinds, 1968b; Altman, 1969), and neurogenesis continues to at least 90 days of age in the mouse (Kaplan and Hinds, 1977). The accessory olfactory bulb, which receives sensory input from the vomeronasal nerve, sits in the caudal

Figure 1. Coronal sections through the olfactory bulb of hamster at one month of age (A and B) and four days of age (C and D). Note the change in size and shape of the bulb between these ages, and the changes in the width and cellular density of the mitral cell layer (MCL) and external plexiform layer (EPL). B and D are higher power views of the MCL and EPL at each age. Abbreviations: EPL= external plexiform layer; Glom= glomerular layer; GrCL= granule cell layer; IPL= internal plexiform layer; MCL= mitral cell body layer; ON= olfactory nerve layer; S= subventricular layer. A,C: bar= 250μ. B,D: bar= 50μ.


ON
Glom
MCL
EPL
IPL
GrCL
A
Glom
EPL
MCL
B
Glom
EPL
IPL
MCL
ON
GrCL
C
Glom
EPL
MCL
D

dorsal part of the bulb. The primary projection neurons from the bulb to olfactory cortex are the mitral cells, and the tufted cells, which are located primarily in the EPL. Classically, the tufted cells have been divided into three categories: inner tufted cells, located in the inner part of the EPL, middle tufted cells, located in the mid- to outer parts of the EPL, and outer tufted cells, which occupy the outer edge of the EPL and the regions bordering the glomeruli (Ramon y Cajal, 1911). The size and shape of the tufted cells are highly variable, but inner tufted cells tend to be about the same size as mitral cells, while outer tufted cells are generally smaller (Haberly and Price, 1977). The tufted cells were originally believed to project to the contralateral olfactory bulb via the anterior limb of the anterior commissure (Ramon y Cajal, 1911). Lohman and Mentink (1969), however, made lesions of the bulb which included either both mitral and tufted cells or tufted cells only, and were able to demonstrate that both cell types project in the LOT to the olfactory cortex. The commissural projections connect the anterior olfactory nuclei of the two sides (Lohman and Mentink, 1969). Retrograde labeling of olfactory bulb cells after HRP injections in olfactory cortex has shown that all three types of tufted cells project to the olfactory cortex, although the proportion of labeled outer tufted cells was usually less than that of inner and middle tufted cells (Haberly and Price, 1977). Tufted cells tend to be labeled more frequently from injections in the olfactory tubercle than from the piriform cortex (Haberly and Price, 1977; Scott et al., 1980). The results of these studies are still consistent with the possibility that some tufted cells, especially outer tufted cells, do not project out of the bulb (Lohman and Mentink, 1969; Haberly and Price, 1977).

Because the gestational period of the hamster (16 days) is considerably shorter than that of the mouse (19 days), we felt it necessary to investigate the time of cell origin in this species. It was conceivable that in the hamster there could be either increased postnatal histogenesis or a more compressed prenatal development. The first experiment reported here defined the times of cell formation of the mitral and tufted cells in the hamster olfactory bulb. The second experiment was designed to determine if the date of cell formation correlates with the time the axon enters and innervates its projection areas. This required the combination of ^{3}H-thymidine labeling for time of cell formation with the retrograde transport of HRP at a time when the olfactory bulb projections are not yet complete. Specifically, the hypothesis tested was that the axons of early-formed cells reach the olfactory cortex before the axons of later-formed cells.

The last experiment proceeds from the results of the first two experiments, to explore the possibility of a developmental principle related to functional recovery and neuroanatomical plasticity in young animals. Devor (1976b) found that when the LOT is cut prior to day 7, axons grow back through the cut and reinnervate the terminal regions. Functional recovery occurs only when the terminal regions are reinnervated (Devor, 1975; Small, 1977). When the LOT is cut after day 7, this reinnervation does not occur, and there is no functional recovery. The extensive literature on regneration (for reviews see: Ramon y Cajal, 1928; Windle, 1956; Bernstein and Goodman, 1973; Guth and Clemente, 1975; Puchala and Windle, 1977) suggests that there are three possible sources of the reinnervating

axons: 1) True regeneration: The regenerative capacities of the CNS following axotomy have been described by Ramon y Cajal (1928). Some fibers proximal to the cut show some regenerative ability, with the formation of a growth cone and subsequent arborization of the growing terminal. The axons grow toward the necrotic zone, but rarely enter either the necrotic zone or the connective tissue scar before the growth is halted and the growing ends are resorbed. The regenerative attempts are much stronger in newborn animals than in the adult. There is evidence for axonal regeneration of the central adrenergic neurons both in the adult (Bjorklund et al., 1971; Stenevi et al., 1973) and younger animals (Nygren et al., 1971), but occurs faster in the younger animals. Regeneration of pryamidal tract axons has been reported after lesions made in the first week of life (Kalil and Reh, 1979). 2) Collateral sprouting: reinnervation could occur by the fibers sprouting from the proximal portion of the transected axons (regenerative sprouting) or by collateral sprouting from nearby uninjured axons. Collateral sprouting from uninjured axons has been reported in many parts of the nervous system (for example: Hicks and d'Amato, 1970; Lynch et al., 1973; Price et al., 1976). Regenerative sprouting from the proximal axons occurs after lesions of the central adrenergic axons (Bjorklund et al., 1971; Nygren et al., 1971). In several systems, sprouting has been found from both the proximal part of the cut axons and from other nearby fibers (adrenergic cells--Pickel et al., 1974; olfactory system--Devor, 1976b; pyramidal tract--Kalil and Reh, 1979). Most of these studies find sprouting to occur either exclusively, or to a much greater extent, in the young animal. 3) Neogenesis: the axons

passing through the cut may represent continued growth of normal, newly-formed axons which had not yet reached the level of the cut at the time of the tract section. A well-documented example of this process is the olfactory nerve, where there is a "reconstitution" of the nerve and its projections by axons of newly-formed receptor cells following olfactory nerve section (Graziadei et al., 1979). Axonal neogenesis as an explanation for CNS axonal regeneration in young animals was first suggested by Ranson (1903). In the visual system, aberrant projections resulting from early lesions appear to be due to continually growing new axons which are no longer receiving the appropriate positional signals (Guillery, 1972; Lund et al., 1973; So, 1979). Since axon section must occur early in the period when the LOT projection is establishing its distribution if functional recovery is to occur, it seemed likely that those fibers which grow through the LOT section prior to day 7 are new axons which had not yet reached the level of the section, rather than regenerating axons or collateral sprouts. The design for the third experiment thus includes ^{3}H-thymidine labeling of the times of formation of the mitral and tufted cells, transection of the LOT at day 3, a recovery period of approximately one month to allow any regrowth and reinnervation of the olfactory cortex projection, and retrograde transport of HRP placed in the projection region caudal to the prior LOT section.

If the hypothesis of continued growth of new axons as the explanation for LOT reinnervation is confirmed, severe limitations are placed on the possibilities for CNS regeneration in the adult. Regardless of the source of the axons, regrowth and reinnervation occur much more easily, and to a greater extent, in young animals

than in the adult. There are critical changes in development that either allow or prevent axonal regrowth, but it is not yet known if these changes are in the cells of origin, or in the tissue into which the axons are growing. It is likely that there are multiple interactions in the development of the system which influence the capacity for regrowth.

CHAPTER II
MATERIALS AND METHODS

Experiment I
Time of Cell Formation

A series of pregnant hamsters (Mesocricetus auratus) was given intraperitoneal injections of ^{3}H-thymidine (5-10μCi/gm) on gestational days 10 (E10), 11, 12, 13, or 14. We did not wish to compromise the pregnancies, so did not inject on E15 or 16. The pups are born after a gestational period of 16 days (E16= P0). Several pups were each injected subcutaneously with ^{3}H-thymidine on postnatal days 1 or 2, but no heavily labeled mitral or tufted cells were seen with these injections, and their data are not included in this report. A summary of the animals from which data were obtained and their specific treatments is given in Table 1.

Animals were mated in the mid-afternoon (E0), thymidine injections were also given mid-afternoon on the days indicated, and birth usually occurred on the morning of E16. The pups were housed with their mothers in solid-bottom cages containing hardwood shavings until the time of sacrifice. At approximately one month of age (see Table 1) the pups were sacrificed by transcardial perfusion of fixative (1% paraformaldehyde, 1.25% glutaraldehyde) for one-half hour, followed by one-half hour of 10% sucrose in 0.1M phosphate buffer. The brains were removed, and the olfactory bulbs were separated from the rest of the brain. The bulbs were rinsed in phosphate buffer for 1-2 hours, then dehydrated in a graded series of methanol and methanol: ethylene

Table 1. Animals and treatments.

Animal number	Day of ^{3}H-thy injection	Total amount ^{3}H-thy(μCi/gm)	LOT section	HRP	Day of sacrifice
1382L (E)[a]	E10	10[b]	--	--	D10
1470L (E)	E10	10[b]	--	--	D49
1471L (E)	E10	10[b]	--	--	D49
1128L (A)	E11	5	--	--	D30
1324L (B)	E11	10	--	--	D26
1325L (B)	E11	10	--	--	D26
1511L (F)	E12	10[b]	--	--	D10
1512L (F)	E12	10[b]	--	--	D10
1560L (F)	E12	10[b]	--	--	D29
1560R (F)	E12	10[b]	--	--	D29
1381L (D)	E13	8[b]	--	--	D10
1468L (D)	E13	8[b]	--	--	D49
1469L (D)	E13	8[b]	--	--	D49
1354L (C)	E14	10	--	--	D36
1354R (C)	E14	10	--	--	D36
1353L (C)	E14	10	--	--	D36
1353R (C)	E14	10	--	--	D36
1546L (I)	E11	10[b]	--	D3	D4
1547L (I)	E11	10[b]	--	D3	D4
1553L (J)	E11	9[b]	--	D3	D4
1554L (J)	E11	9[b]	--	D3	D4

Table 1--continued.

1502L (H)	E13	8[b]	--	D3	D4
1503L (H)	E13	8[b]	--	D3	D4
1505L (H)	E13	8[b]	--	D3	D4
1556L (K)	E13	8[b]	--	D3	D4
1487L (D)	E13	8[b]	--	D53	D54
1601L (H)	E13	8[b]	--	D30	D31
1687L (K)	E13	8[b]	--	D36	D37
1749L (L)	E13	8[b]	--	D26	D27
17501 (L)	E13	8[b]	--	D26	D27
1670L (J)	E11	9[b]	D3	D34	D35
1674L (J)	E11	9[b]	D3	D37	D38
1675L (J)	E11	9[b]	D3	D37	D38
1684L (J)	E11	9[b]	D3	D39	D40
1579L (G)	E13	8[b]	D3	D30	D31
1580L (G)	E13	8[b]	D3	D30	D31
1743L (L)	E13	8[b]	D3	D25	D26
1747L (L)	E13	8[b]	D3	D26	D27

[a]Litter identification.

[b]In two doses, one hour apart.

glycol monomethyl ether (1:1), cleared in methyl benzoate until sinking and in toluene (5-10 minutes only), then embedded in Paraplast Plus. The tissue was processed in this manner rather than the conventional formalin fixation and ethanol dehydration to correspond with the brains in Experiments II and III. Ethanol dehydration of brains fixed with the paraformaldehyde-glutaraldehyde mixture resulted in very brittle, over-hardened tissue.

Coronal sections were cut at 8μ through the entire olfactory bulb. Serial sections were mounted onto acid-cleaned chrom-alum slides, deparaffinized, and prepared for autoradiography with Kodak NTB-3 emulsion. The slides were allowed to expose for 8 weeks at $4^{o}C$, then developed in Kodak D-19 developer and lightly counterstained with cresyl violet or neutral red. Exposure times of 3-6 weeks were considered initially, but the labeling was generally inferior compared to the 8 week exposure time.

Experiment II
Correlation of Cell Birthdate and Axonal Outgrowth

For this experiment, we chose two days for ^{3}H-thymidine injections: one early in the period of mitral and tufted cell formation (E11), and one late in this period (E13), as determined by the results of Experiment I. On E10 so few mitral or tufted cells were labeled that the probability of finding double-labeled cells would be extremely low. On E14 mostly outer tufted cells were labeled, and it is not certain if all of these cells send efferent projections to the olfactory cortex (Lohman and Mentink, 1969; Haberly and Price, 1977). Horseradish peroxidase (Sigma, Type VI) was placed in the olfactory

tubercle, LOT, and/or piriform cortex of animals in both groups on day 3 postnatally. Several animals with ^{3}H-thymidine injections on E13 received HRP injections at about one month of age (see Table 1).

For the HRP placement, a small pellet of 80% HRP in saline was dried onto the tip of a 50μ diameter wire. One eye was removed and a small hole was made in the medial wall of the orbit, and the tip of the wire containing the HRP was placed in the desired region under direct visualization. In the older animals it was most efficient to make a small slit in the pia and underlying LOT before inserting the HRP. The insertion wire was left in place for 2-3 minutes to allow the HRP to dissolve away from it, then gently removed. The animals 3 days of age were anesthetized by immersion in crushed ice; the older animals were anesthetized by intraperitoneal injection of Chloropent, .0038ml/gm. Bleeding was controlled as necessary with saline- or thrombin-soaked Gelfoam. The wounds were closed with Steristrips or sutures, and the skin was carefully cleaned to reduce post-surgical cannibalism by the mother. When all pups in a litter had recovered from the anesthesia, the younger pups were returned to the mother. The older animals were housed separately after surgery. All animals were allowed to survive for 24 hours after the HRP placement.

The animals were sacrificed as in Experiment I. After removal from the skull, the olfactory bulbs were separated from the rest of the brain and processed separately. The brains were immersed in sucrose-buffer overnight at 4^{o}C, then embedded in gelatin-albumen with glutaraldehyde (0.5ml glutaraldehyde per 10ml gelatin-albumen) for 2-4 hours. Frozen sections were cut on a sliding microtome at

50μ in the coronal plane. To determine the location of the HRP injection site, equidistant sections were reacted with either tetramethylbenzidine (TMB; Mesulam, 1978) or diaminobenzidine (DAB) with cobalt chloride enhancement (Adams, 1977) and counterstained with neutral red.

To identify retrogradely-labeled cells, the olfactory bulbs were reacted with DAB, en bloc, as described below (modified from Moody and Heaton, 1981). The times indicated for each step are for 4 and 30 day old olfactory bulbs, respectively. The en bloc reaction works well in embryonic and neonatal tissue, but the DAB does not penetrate older tissue well, requiring extremely long reaction times. The size of the tissue block did not seem to be a major factor, within the range of this experiment. The tissue should be agitated during all steps, when possible.

1) Rinse twice in phosphate buffer, pH 7.3 (15/30 min each).
2) Rinse in 0.1M Tris-HCl buffer, pH 7.6 (15/30 min).
3) 0.5% $CoCl_2$ in Tris buffer (60min/2hr).
4) Rinse twice in 0.1M Tris buffer (15/30 min each).
5) Rinse in 0.1M phosphate buffer, pH 7.3 (15/30 min).
6) 0.05-0.1% DAB, in phosphate buffer, at $4^{o}C$ (60min/3hr).
7) 0.01-0.02% H_2O_2 in DAB (60min/4hr or more).
8) Rinse twice in phosphate buffer (15/30 min each).

The tissue was then dehydrated and embedded, and prepared for autoradiography as in Experiment I.

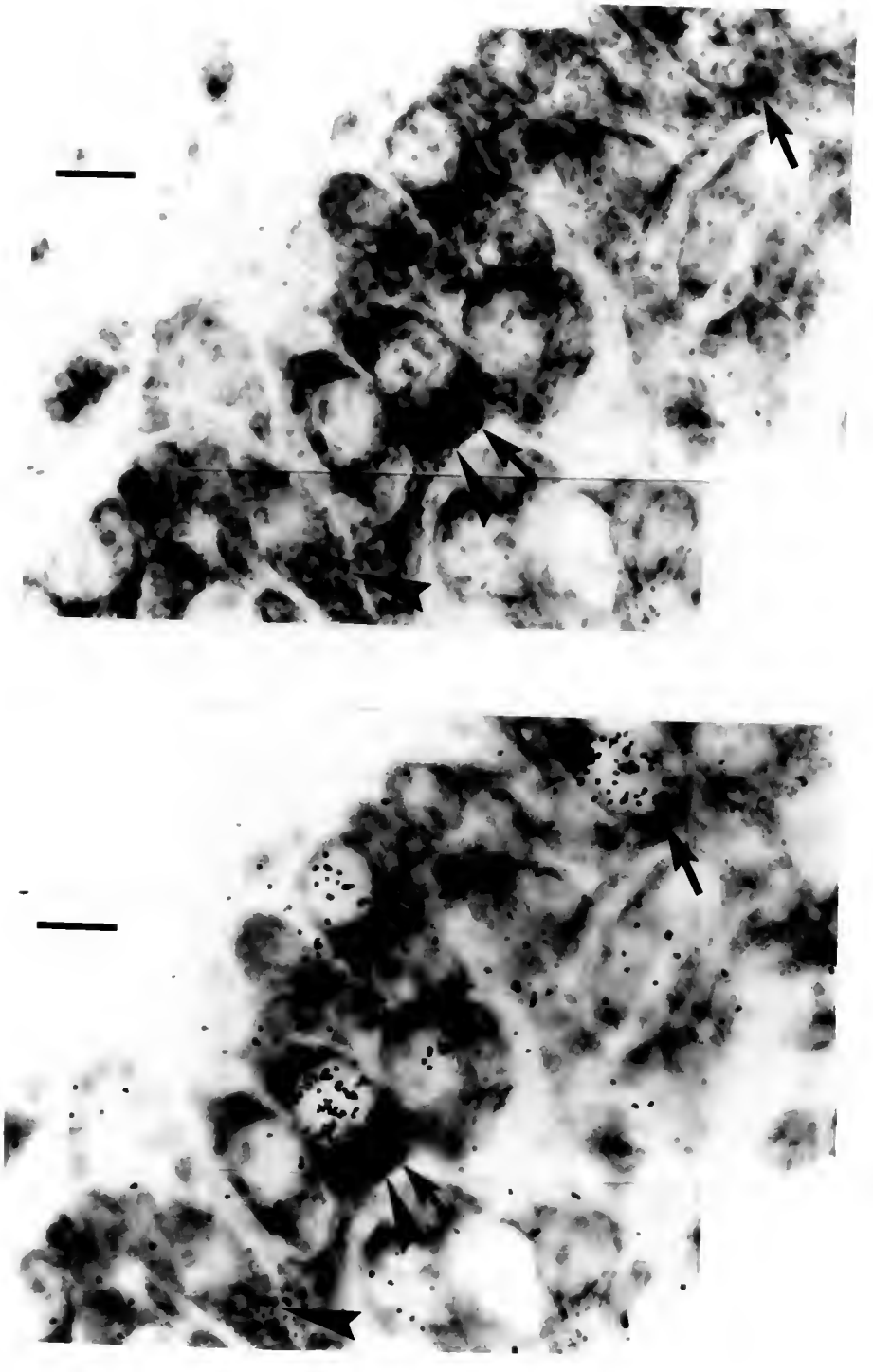
Experiment III
Early Transection of the Lateral Olfactory Tract

Animals which had received ^{3}H-thymidine on E11 or E13 were given a transection of the lateral olfactory tract on day 3 postnatally. The approach for the surgery was the same as that described for HRP placement into this region in the previous experiment. The region around the LOT was exposed, and a small cut through the LOT was made using a microknife, taking care not to cause unnecessary additional damage. After surgery the pups were returned to the mother for 3-4 weeks, an interval more than sufficient to allow reinnervation of the olfactory cortex by the olfactory bulb efferents (Devor, 1976b; Small, 1977). When the pups were about a month old, they received injections of HRP into the olfactory projection areas caudal to the prior LOT section. The location of the LOT section was identified first by scar formation on the skull, and then by direct visualization of the residual portion of the LOT rostral to the cut. The LOT was not visible as a distinct bundle caudal to a complete transection. The animals were sacrificed and the tissue reacted with DAB and processed for autoradiography as described in the two previous experiments. Because of the multiple procedures combined in these experiments, large numbers of animals were used to produce the groups from which data were obtained, as listed in Table 1. The animals from which data are reported are only those with adequate ^{3}H-thymidine labeling, histologically verified complete LOT sections, and satisfactory HRP injections in the olfactory projection areas.

Data Collection and Analysis

Cmaera lucida drawings were made of olfactory bulb sections at a magnification of 125X. Mitral and tufted cells were identified on the basis of their size, large nucleus and abundant cytoplasm, and position in the MCL or EPL. No distinction was made between the different types of tufted cells. Only cells whose nucleus was clearly in the plane of section were counted. The sections were examined under the microscope at 600X for the presence of cells labeled with ^{3}H-thymidine and/or HRP (Figure 2). Cells which had many silver grains over the nucleus (about 5 times background or more) were considered "heavily-labeled" with thymidine and were presumed to have undergone their last division on the day of ^{3}H-thymidine injection. A thorough discussion of the problems in defining "Heavily-labeled" cells can be found in Sidman (1970). The HRP reaction product is seen as a dark brown, particulate substance in the cytoplasm of the cell body and proximal dendrites. The two labels are easily distinguished under the microscope on the basis of their locations in the cell (^{3}H-thymidine in the nucleus, HRP in cytoplasm), color and size (^{3}H-thymidine is small, distinct black grains, HRP is larger, dark brown particles), and plane of focus (silver grains for ^{3}H-thymidine are in the emulsion above the section, HRP reaction product is within the cell). The positions of ^{3}H-thymidine and HRP-labeled cells were indicated on the camera lucida drawings. Labeled granule cells and glia were not included on the drawings. For each olfactory bulb analyzed in Experiment I, five sections were drawn: rostral OB, two sections in mid-OB, caudal OB, and a section which contained the accessory olfactory bulb. In Experiments II and III, two sections in the

Figure 2. High power photomicrograph of cells in the mitral cell layer of an animal injected with ^{3}H-thymidine on E11, HRP on day 3. The plane of focus in the upper half of the figure is through the emulsion above the tissue, while that in the lower half is through the tissue itself. The ^{3}H-thymidine label is seen as silver grains in the emulsion (upper). Heavily-labeled cells have many silver grains above their nuclei. The HRP reaction product is a particulate substance in the cytoplasm (lower). There are many unlabeled cells, a cell labeled with ^{3}H-thymidine only (arrow, lower left), a cell with HRP label only (arrowhead, upper right), and one double-labeled cell (arrow and arrowhead). DAB reaction, counterstained with neutral red. Bar= 10μ.



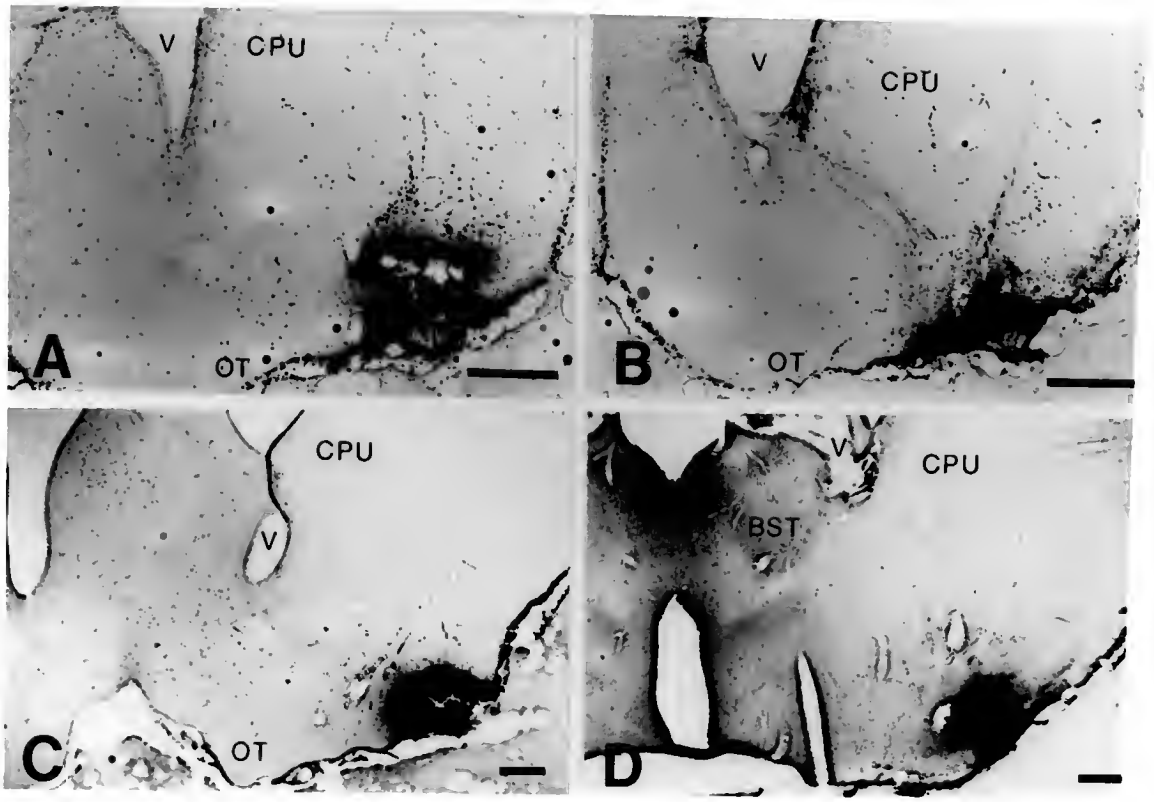
mid-OB were drawn for each brain, since no rostral-caudal differences were found in Experiment I.

The position of each labeled cell within the EPL was determined in the following manner. The distance from the outer edge of the MCL to the labeled cell and the distance from the MCL to the inner edge of the glomerular layer were measured. The ratio (r) of these distances was calculated. An r of 0 indicates a cell in the MCL, an r of 1.0 a cell in the glomerular layer (see figure 5). For each section drawn, the mean r ($\bar{r}$) and mean EPL depth were calculated. An overall $\bar{r}$ was then determined for each olfactory bulb.

Additional measurements were made on the sections from Experiments II and III, and on the mid-OB sections from the E11 and E13 brains in Experiment I. The total number of all mitral and tufted cells per section was counted (both labeled and unlabeled cells), and the perimeter of the outer edge of the MCL was measured. The numbers of mitral cells per unit perimeter, tufted cells per perimeter, and total cells per perimeter were calculated, and the ratio of mitral to tufted cells determined. The proportion of ^{3}H-thymidine-labeled cells to total mitral and tufted cells was also calculated for these sections (p(thy)).

The location and extent of the site of HRP placement was determined for each animal in Experiments II and III (Figure 3). Cell counts for ^{3}H-thymidine-labeled and total cells, as well as HRP-labeled cells,were made only in brains which had HRP injections in the olfactory projection regions which were of a size sufficient to produce retrograde labeling of a reasonable number of olfactory bulb cells (Grafe and Leonard, 1981). In Experiment III, the site

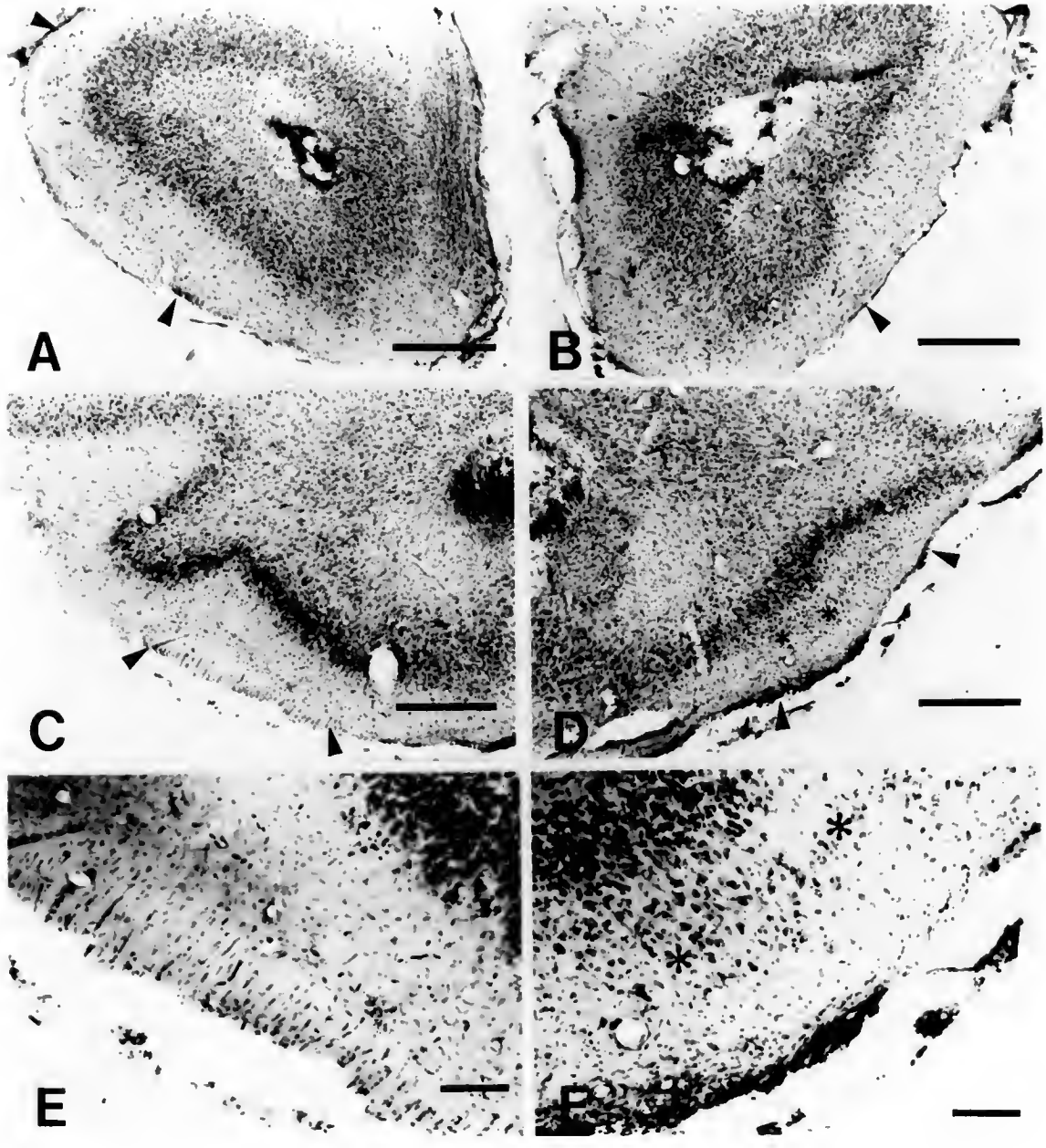
Figure 3. Examples of HRP placement sites for Experiments II and III. A. Animal 1553; ^{3}H-thymidine E11, HRP D3. HRP placement in ventral piriform cortex and just deep to the lateral olfactory tract (LOT), at the level of the caudal olfactory tubercle. B. Animal 1556; ^{3}H-thymidine E13, HRP D3. HRP placement in and surrounding LOT, at a more rostral level than in A. C. Animal 1674; ^{3}H-thymidine E11, LOT D3, HRP at one month. HRP placement in and surrounding LOT, at the level of mid-olfactory tubercle. D. Animal 1743; ^{3}H-thymidine E13, LOT D3, HRP at one month. HRP placement in caudal piriform cortex. Abbreviations: BST= bed nucleus of the stria terminalis; CPU= caudate-putamen; OT= olfactory tubercle; V= lateral ventricle. All brains reacted with DAB; D is lightly counterstained with neutral red, others have no counterstain. Bars= 500μ.


V
CPU
A
OT
V
CPU
B
OT
CPU
V
C
OT
V
CPU
BST
D

of LOT transection was verified in histological sections, and only those animals with complete tract sections were included in the analysis. Caudal to the level of the cut, the LOT was completely absent as a bundle on the surface of the brain, and there was usually some distortion of the underlying cortex at the cut (Figure 4).

For Experiments II and III, approximately 200 (Experiment II) to 400 (Experiment III) sections from each olfactory bulb were examined under the microscope at 600X for the presence of HRP-labeled and double-labeled cells. Using the total number of cells per section counted as above, the proportion of HRP-labeled cells to total cells was calculated (p(HRP)). If there was no interaction between the presence of ^{3}H-thymidine and that of HRP in the cells, the incidence of both labels in the same cell can be predicted, based on the proportions of each label individually. The predicted proportion of double-labeled cells for each animal is calculated by multiplying the proportion of HRP-labeled cells by the proportion of ^{3}H-thymidine-labeled cells: p(double-labeled, predicted) = p(HRP) X p(thy). This was compared to the actual number of double-labeled cells identified. Deviation from the predicted value indicates some interaction of the presence of ^{3}H-thymidine label (time of cell formation) and HRP label (time the axon reaches the projection areas). If the number of double-labeled cells is greater than predicted, there is a positive correlation between the particular time of cell formation and the time of axonal outgrowth. Fewer than predicted double-labeled cells indicates a negative correlation between these events.

Figure 4. Site of lateral olfactory tract (LOT) transection. Animal 1743, LOT D3, sacrificed at one month of age. A, C, and E are from the right (normal) side of the brain. B, D, and F are from the left (operated) side. A and B. Sections through the olfactory peduncle, rostral to the site of LOT transection. The LOT can be seen between the two arrowheads. In A, the normal LOT is a pale region on the surface of the brain, easily identified by the rows of glial nuclei. In B, the LOT rostral to the transection is somewhat reduced, and the glial nuclei are more disordered. C-F. Sections through the rostral olfactory tubercle, at the level of the cut. In C, the LOT is a discrete bundle of fibers (between arrowheads). E. Higher power view of region between arrowheads in C. D. The LOT is completely absent on the operated side. There is some glial scarring near the pial surface, and some aberrant cell groups are seen deep to where the LOT would be found (asterisks). F. Higher power view of region between arrowheads in D. All sections counterstained with neutral red. A-D: bars= 500μ. E,F: bars= 100μ.


A
B
C
D
E
F

CHAPTER III
RESULTS

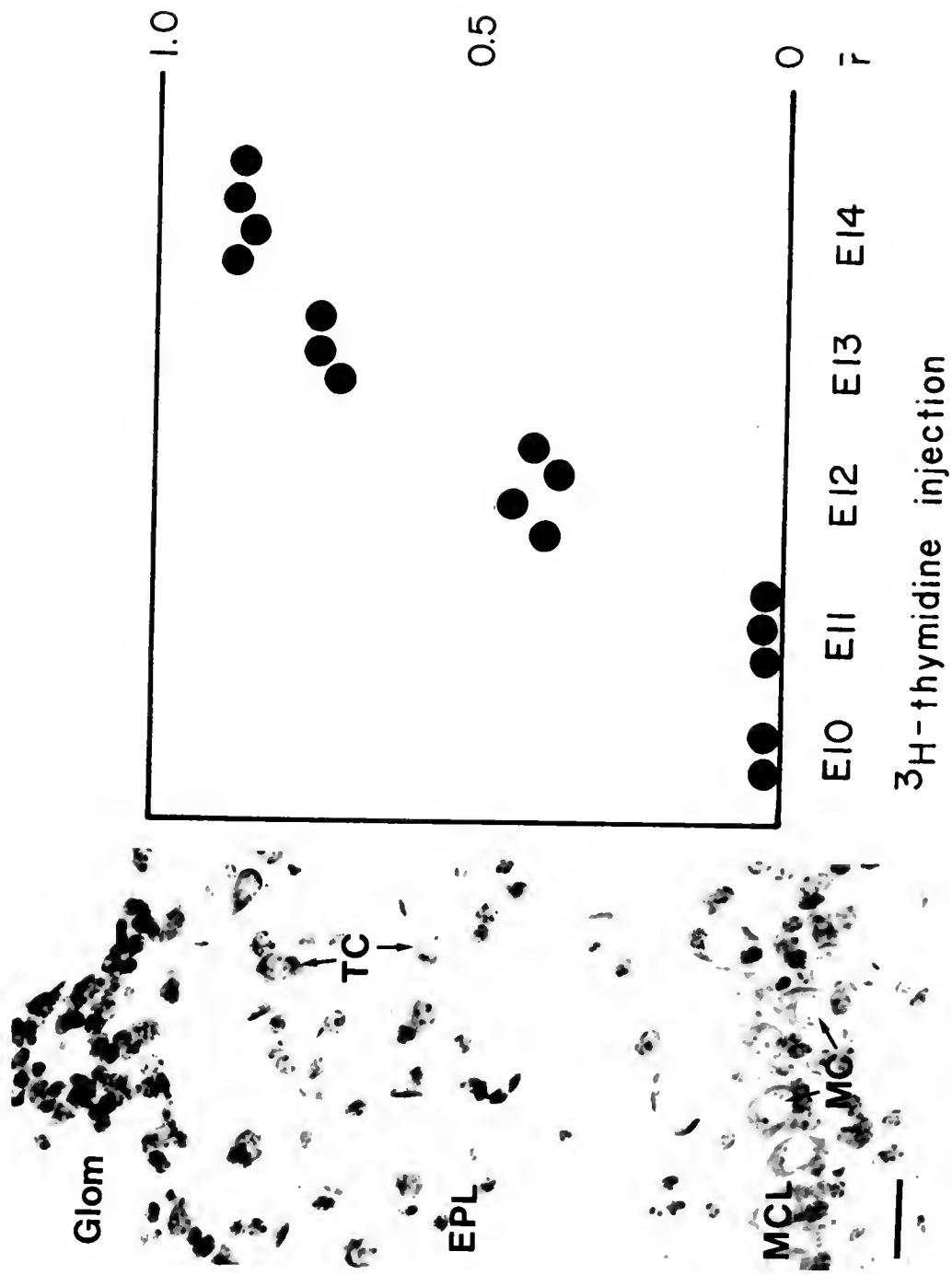
Experiment I

Results

Heavily-labeled mitral and tufted cells were found with ^{3}H-thymidine injections on E10, 11, 12, 13, and 14, but not on P1 or P2. Following ^{3}H-thymidine injection on E10, only a very few mitral cells were heavily labeled (5 cells in 14 sections, 3 animals). Mitral cells apparently undergo their last division primarily on E11 and E12. No labeled mitral cells were seen following injections on E13 or E14. Tufted cells were formed on E11 to E14. Inner tufted cells were formed predominantly on E11 and E12, middle tufted cells on E12 and E13, and outer tufted cells on E13 and E14. We did not inject animals on E15 or 16 (day of birth), so the length of the period of outer tufted cell formation is uncertain.

The depth analysis shows that there is an outward progression of the position of cells formed from E10 to E14 (Figure 5). The overall $\bar{r}$ values for each date of injection are E10= 0; E11= .03 (n= 25-37, S.E.M.= .003-.020); E12= .39 (n= 164-208, S.E.M.= .024-.028); E13= .72 (n= 93-219, S.E.M.= .012-.023); E14= .86 (n= 90-152, S.E.M.= .011-.014). At each age there was a range of cell positions, but the overall variability was quite low. In Figure 5, the standard errors of the means are within the boundaries of the circles for all animals. We found no consistent regional differences

Figure 5. Left. Photomicrograph of MCL and EPL of hamster olfactory bulb, showing locations of mitral and tufted cells. Abbreviations: EPL= external plexiform layer; Glom= glomerular layer; MC= mitral cell; MCL= mitral cell body layer; TC= tufted cell. Bar= 25μ. Right. Representation of MCL and EPL, indicating average positions ($\bar{r}$) of heavily-labeled cells following ^{3}H-thymidine injections on E10 to E14. All animals were sacrificed at about one month of age. Each circle represents $\bar{r}$ for one animal (SEM is within the boundaries of each circle for this experiment). See text (methods) for explanation.


1.0
0.5
0
r̄
E10
E11
E12
E13
E14
3H-thymidine injection
Glom
TC
EPL
MCL
MC

within the bulb in the times of mitral and tufted cell formation, but our sample size may have been too small to demonstrate subtle differences.

Experiment II

Rationale

Animals with ^{3}H-thymidine injections on E11 or E13 were each given injections of HRP into the olfactory bulb projection areas on day 3 postnatally. The numbers of mitral and tufted cells with ^{3}H-thymidine label, HRP label, and both thymidine and HRP labels, and the total numbers of unlabeled mitral and tufted cells were counted. At day 3, the efferent projections from the bulb are still incomplete (Leonard, 1975; Schwob and Price, 1978). The HRP injection on day 3 will label the olfactory bulb cells whose axons enter the lateral olfactory tract and innervate the terminal regions early in the course of development of this projection. If there is no interaction between the time of cell formation and the time the axon reaches the projection areas, the proportion of cells with both ^{3}H-thymidine and HRP label would be equal to the proportion of HRP-labeled cells multiplied by the proportion of ^{3}H-thymidine-labeled cells. Our hypothesis was that the earlier-formed cells send out their axons earlier, thus we predicted that there would be more double-labeled cells in the E11 group than in the E13 group.

Results

Table 2 indicates for each animal the number and percent of HRP and ^{3}H-thymidine-labeled cells, and the predicted and actual proportions of cells containing both labels. For each animal in the

Table 2. Numbers of cells labeled with HRP and ^{3}H-thymidine after HRP placement on day 3.

Treatment[a]	Brain #	HRP-labeled cells #/section (%)	^{3}H-thy-labeled cells #/section (%)	Double-labeled cells: %x10^{2} Predicted	Actual	Actual vs. Predicted
E11/HRP D3	1546L	21.2 (5.9)	7.3 (2.0)	10.0	20.0	>
	1547L	9.2 (2.8)	6.0 (1.8)	5.0	9.0	>
	1553L	31.1 (7.3)	22.0 (5.0)	35.0	67.0	>
	1554L	30.7 (7.3)	18.0 (4.0)	28.0	74.0	>
E13/HRP D3	1502L	3.7 (0.8)	18.0 (3.7)	3.0	0.3	<
	1503L	4.0 (0.8)	22.0 (4.9)	3.9	1.2	<
	1505L	2.8 (0.6)	30.5 (6.8)	4.2	0.9	<
	1556L	23.1 (5.0)	28.0 (5.6)	26.0	0	<

[a]Treatment= age at thymidine injection/ age at HRP placement.

E11, HRP D3 group, the actual number of double-labeled cells is about twice the predicted value. In the E13, HRP D3 group, three animals had fewer than predicted double-labeled cells (about one-fifth the predicted value), and one animal had no cells containing both labels. The presence of double-labeled cells in brains of the E13 group which had HRP injections at one month of age confirmed that cells formed on E13 send their axons into the projection areas and maintain these connections. These results support the hypothesis that the time of axonal innervation of terminal regions correlates with the time of cell birth.

A comparison of the depth distribution of ^{3}H-thymidine-labeled cells between the animals in this experiment sacrificed on day 4 and those in this and the previous experiment sacrificed at one month shows that $\bar{r}$ is significantly lower in the E13, D4 group than E13, 1 month (Figure 6; .59 versus .72, $t = 3.26$, $p < .005$). There was no difference in $\bar{r}$ between E11, D4 and E11, 1 month ($\bar{r} = .04$, $\bar{r} = .03$, respectively). The cells formed on E13 have apparently not yet reached their final positions in the EPL by day 4, while those formed on E11 have already completed their migration.

When all the olfactory bulb projection neurons (labeled and unlabeled) are considered, the ratio of all mitral cells to tufted cells also changes significantly between 4 days and one month. The ratio of mitral to tufted cells for ^{3}H-thymidine E11 animals is 4.14 at day 4, 0.74 at one month ($t = 13.1$, $p < .001$). The ratio for ^{3}H-thymidine E13 animals is 6.23 at day 4, 0.72 at one month ($t = 16.4$, $p < .001$). Figure 7 shows that at day 4 there are many more mitral than tufted cells, but by one month this ratio is reversed so that there are more tufted than mitral cells. This reflects the fact that the

Figure 6. Positions of heavily-labeled cells following ^{3}H-thymidine injections on E11 or E13. Animals were sacrificed at either 4 days of age, or about one month. Each circle represents $\bar{r}$, $\pm$ SEM for one animal. The value of $\bar{r}$ for E13, D4 is reduced compared to that for E13, 1 month. Details are in text.

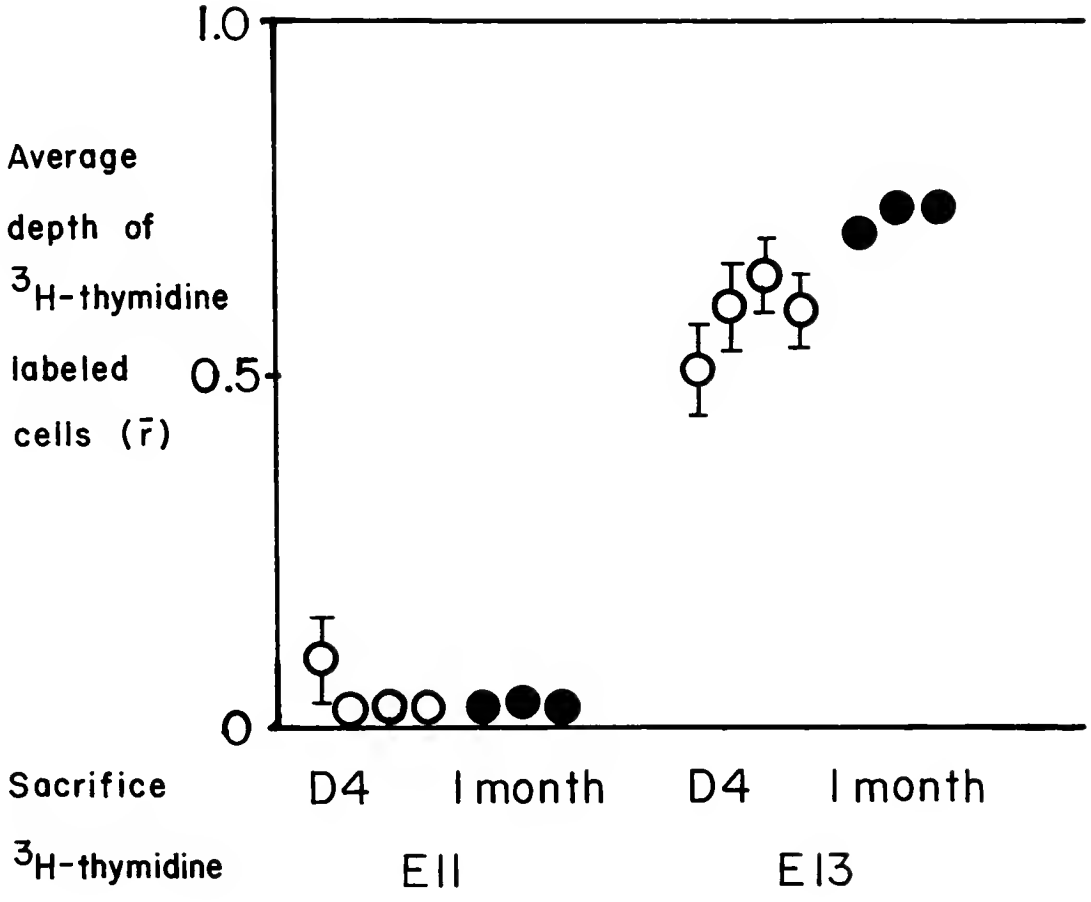
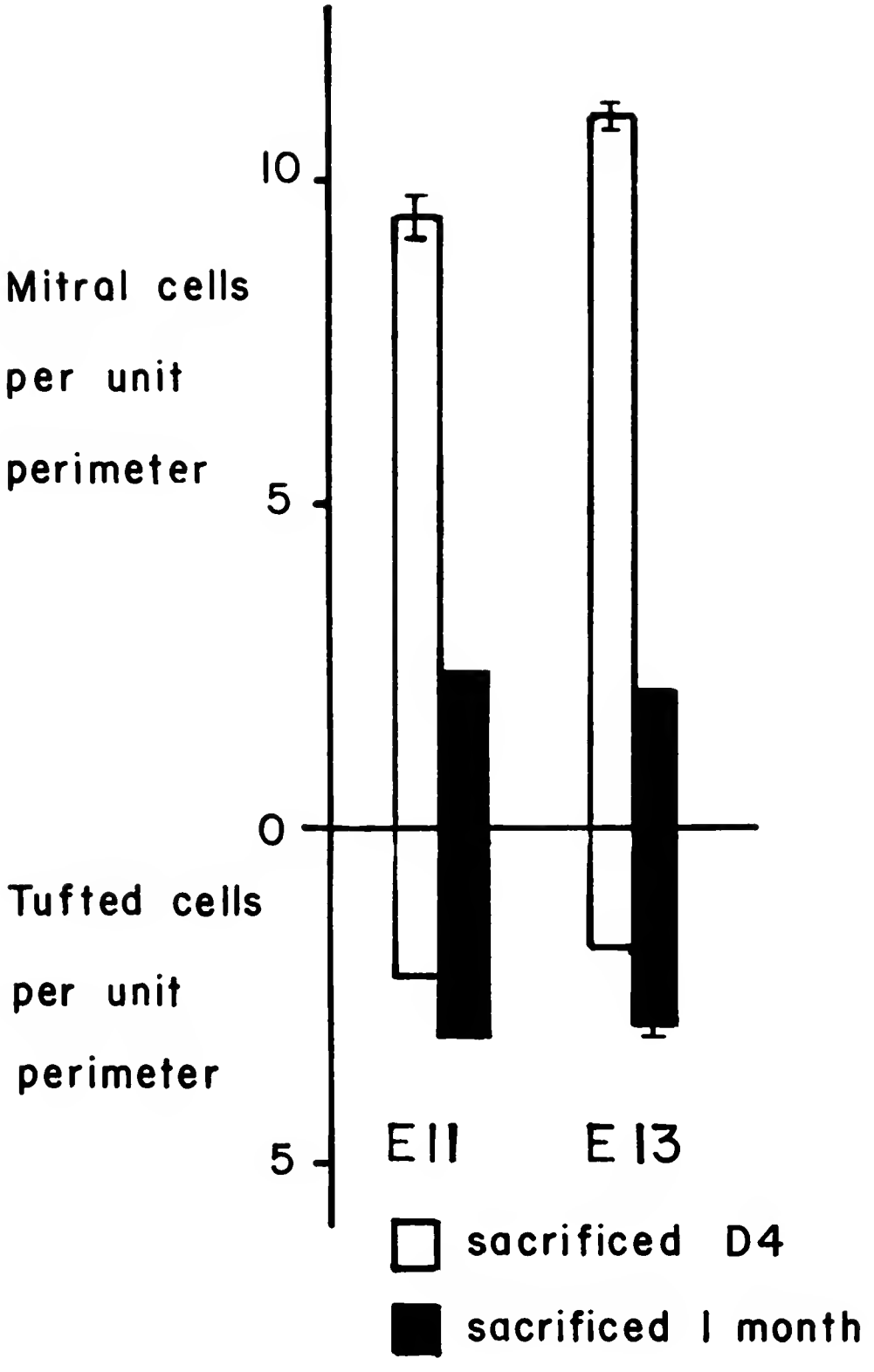

1.0
Average
depth of
^{3}H-thymidine
labeled
cells ($\bar{r}$)
0.5
0
Sacrifice
^{3}H-thymidine
D4
1 month
D4
1 month
E11
E13

Figure 7. Change in the ratio of mean total number, ± SEM (labeled and unlabeled) of mitral to tufted cells between 4 days of age and one month. Animals received injections of ^{3}H-thymidine on E11 or E13. At day 4, there are many more mitral than tufted cells, but the ratio is reversed at one month. At 4 days of age, the number of cells in the mitral cell layer is greater in the E13 animals than the E11 animals.


10
Mitral cells
per unit
perimeter
5
0
Tufted cells
per unit
perimeter
5
E11
E13
sacrificed D4
sacrificed 1 month

MCL is several cells thick at 4 days of age, but thins to a single cell layer by one month (see Figure 1). The changes in the direction of the ratio indicate that some cells in the MCL at day 4 are no longer in that position at one month, and the thinning of the MCL is not simply due to an expansion of the perimeter of the bulb. Surprisingly, at 4 days of age, there are more total cells present in the MCL if the animal received a ^{3}H-thymidine injection on E13 rather than E11. The injection of ^{3}H-thymidine on E13 affects the positions of cells in (or passing through) the MCL on day 4. This effect will be discussed later in relation to the consequences of LOT section in Experiment III.

Experiment III

Rationale

Transection of the LOT at 3 days of age allows some bulb efferents to reinnervate the olfactory cortex caudal to the cut, and the density of innervation rostral to the cut is increased (Devor, 1976b). Our hypothesis was that the fibers which grow through the cut are new fibers which had not yet reached the level at which the tract was sectioned. Experiment II demonstrated that the earlier-formed cells send their axons into the projection areas first. For this experiment, the assumption is that the later-formed cells send their axons out last--a hypothesis which could not be directly tested in Experiment II, since it is not possible to selectively label the later-arriving axons with HRP in an intact animal. The axons of cells formed on E11 are in (and may be expanding within) their projection regions by day 3. Very few axons of cells formed on E13 have reached this level by day 3. By adding a tract section on day 3, the

early-arriving axons would be cut. If the late-arriving axons grow through the cut, these fibers and their cells of origin will be preferentially labeled by HRP injection in the olfactory cortex one month later. If our hypothesis is to be supported, the number of cells labeled with both ^{3}H-thymidine and HRP should be much greater in the group with ^{3}H-thymidine injection on E13 than on E11.

Results

Our results do not support the hypothesis. They demonstrate, instead, that cells formed on E11, but not on E13, are able to reinnervate the olfactory cortex (Table 3). Two of the E11 animals had more double-labeled cells than would be predicted based on a random association of the birthdate and axonal uptake of HRP. One animal had fewer than predicted double-labeled cells, while the fourth animal had no double-labeled cells. None of the animals that received ^{3}H-thymidine injections on E13 had any double-labeled cells. Few cells formed on E13 were projecting through the level of the tract section at day 3 (the time of the cut), and none passed through the cut and subsequent scar.

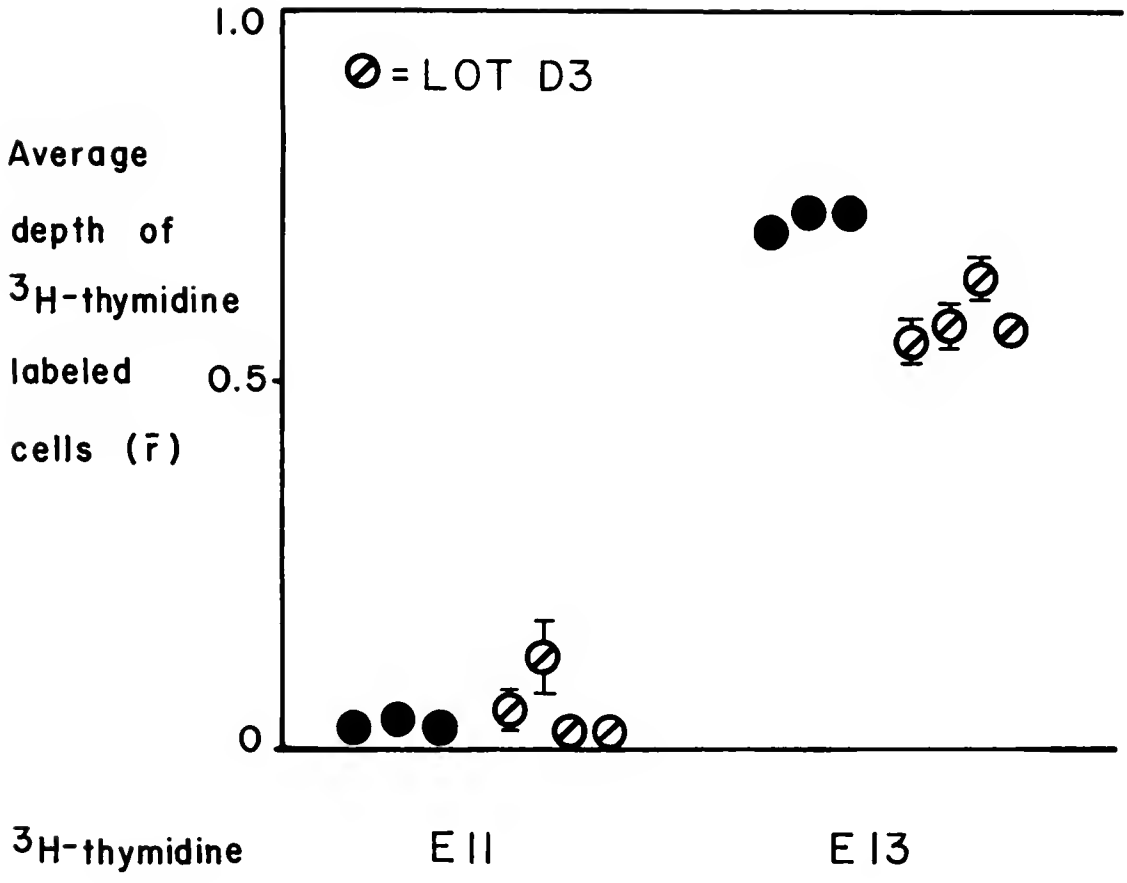
Several other effects of LOT section on day 3 were observed. Tract section on day 3 in animals with ^{3}H-thymidine injections on E13 produced a decrease in the average depth of ^{3}H-thymidine-labeled cells in the EPL, as compared to animals with no tract section (Figure 8). The value of $\bar{r}$ in LOT-section animals is 0.59, as compared to 0.72 for intact animals ($t = 4.67$, $p < .001$). The cells formed on E13 do not move as far into the EPL after tract section as they do in the normal animal. There was no change in the $\bar{r}$ of labeled cells in the animals with tract sections injected with ^{3}H-thymidine on E11.

Table 3. Proportions of double-labeled cells after LOT transection.

Treatment[a]	Brain #	Double-labeled cells: %x10^2 Predicted	Actual	Actual vs. Predicted
E11/	1670L	1.6	0.3	<
LOT D3/	1674L	0.4	0.9	>
HRP	1675L	0.1	0	<
	1684L	0.8	1.4	>
E13/	1579L	2.2	0	<
LOT D3/	1580L	4.8	0	<
HRP	1743L	0.04	0	<
	1747L	0	0	-

[a]Treatment= age of ^{3}H-thymidine injection/ age of LOT transection/ HRP placement at one month of age.

Figure 8. Positions of heavily-labeled cells after transection of the LOT at day 3 (LOT D3). Animals received injections of ^{3}H-thymidine on E11 or E13. All animals were sacrificed at about one month of age. Each circle represents $\bar{r}$, $\pm$ SEM for one animal. The value of $\bar{r}$ is reduced following LOT section in the E13 animals.


1.0
⊘ = LOT D3
Average
depth of
3H-thymidine
labeled
cells (r̄)
0.5
0
3H-thymidine
E11
E13

Both the E11 and E13 ^{3}H-thymidine groups have a decreased number of total (unlabeled) mitral cells per unit perimeter following LOT section (Figure 9). The mitral cells are formed early (E11 and E12), and are likely to have had their axons severed by the LOT section. The number of tufted cells is increased in the E13 ^{3}H-thymidine animals, but not in the E11 animals. Recall that animals in Experiment II injected with ^{3}H-thymidine on E13 and sacrificed on day 4 had more cells in the MCL than E11 ^{3}H-thymidine animals. The injection of ^{3}H-thymidine on E13 appears to have an effect on the positions of cells destined for the EPL that have yet to undergo their final division. Examination of the numbers of ^{3}H-thymidine-labeled cells in brains with and without tract sections reveals that there is no loss of either E11 ^{3}H-thymidine-labeled cells or E13 ^{3}H-thymidine-labeled cells. The loss of mitral cells following LOT section is apparently due to the loss of cells which were born between these two injection times, or on E12, broadly speaking.

Tract section also had no effect on the mean width of the EPL (Figure 10). A normal, age-related change in width occurs between day 4 and one month, but this increase was not affected by tract section on day 3.

Figure 9. The ratio of total mitral to tufted cells in animals with or without day 3 LOT section (LOT D3). Animals were injected with ^{3}H-thymidine on E11 or E13, and all were sacrificed at about one month of age. Both E11 and E13 groups have a decreased number of mitral cells, and the E13 animals have an increased number of tufted cells after LOT section.

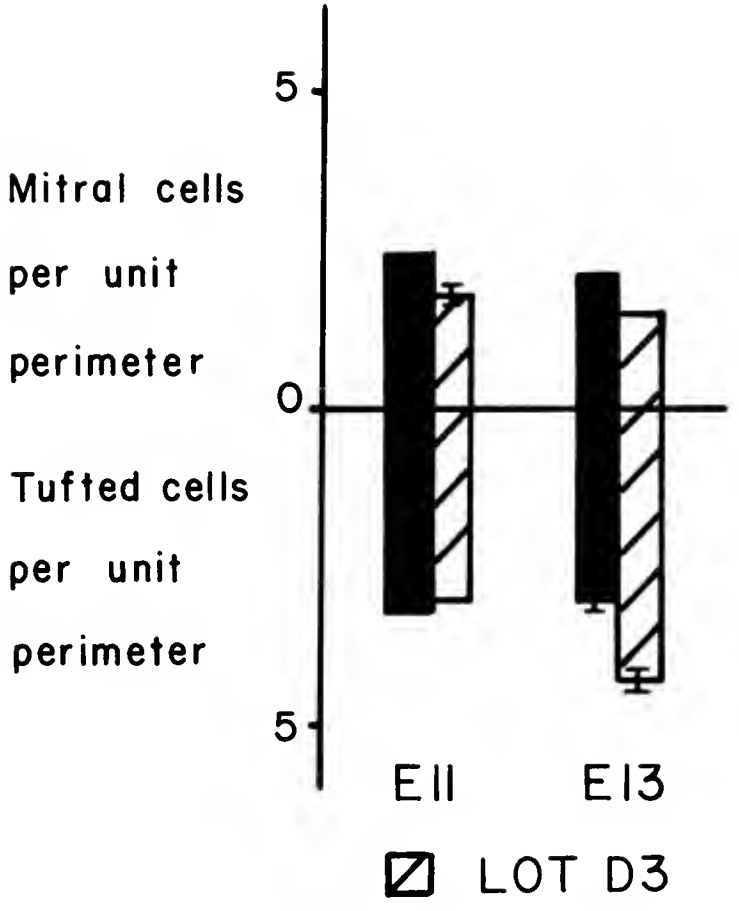
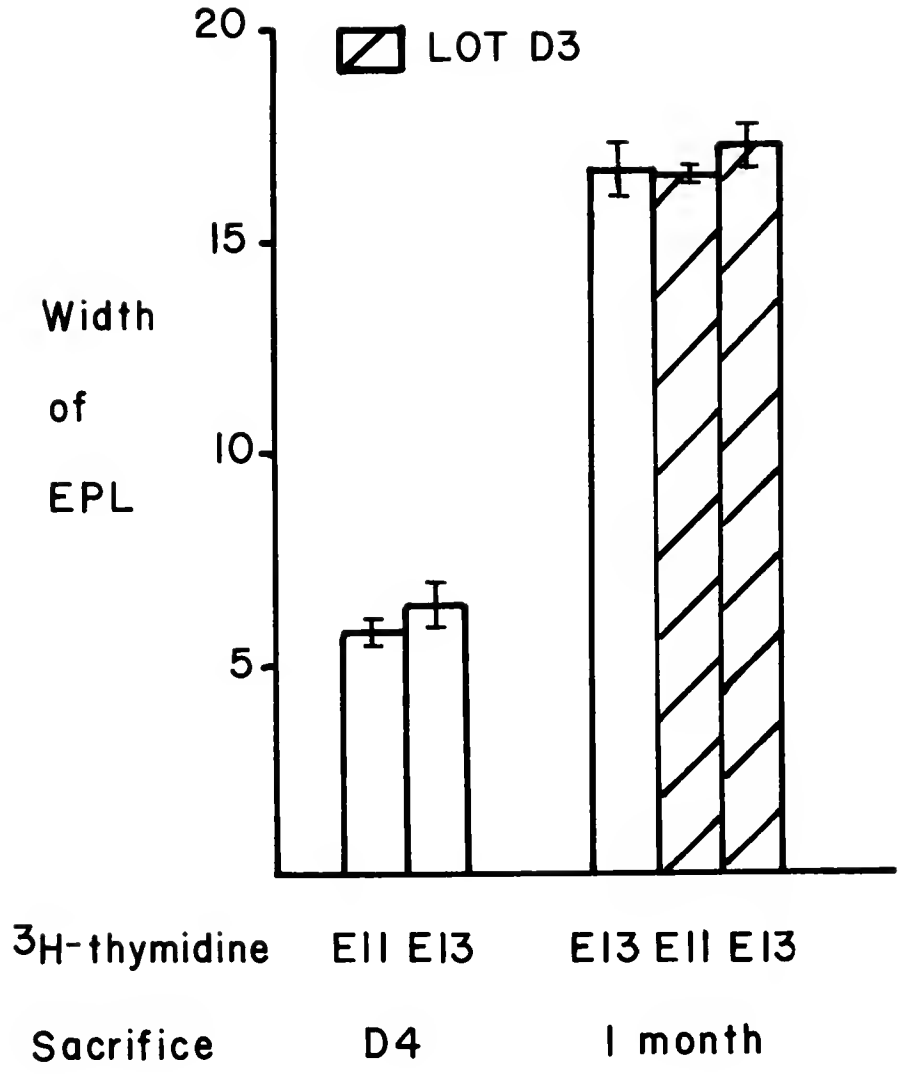

5
Mitral cells
per unit
perimeter
0
Tufted cells
per unit
perimeter
5
E11
E13
LOT D3

Figure 10. Mean width, ± SEM, of the EPL in animals injected with ^{3}H-thymidine on E11 or E13 and sacrificed at day 4 or one month. Tract section at day 3 (LOT D3) had no effect on the width of the EPL.


20
15
10
5
LOT D3
Width
of
EPL
3H-thymidine
E11 E13
E13 E11 E13
Sacrifice
D4
1 month

CHAPTER IV
DISCUSSION

Time of Mitral and Tufted Cell Formation

The mitral and tufted cells of the hamster olfactory bulb undergo their last divisions between E10 and E14. Mitral cells are formed primarily on E11 and E12, and tufted cells are formed on E11 to E14. In the mouse olfactory bulb (Hinds, 1968a) mitral cells are formed on E10-E15, peaking at E13, and tufted cells are formed on E10-E18, peaking at E16. The period of histogenesis in the hamster is both earlier and more compact than that of the mouse. The golden hamster is born after an extremely short gestational period of 16 days. This is one of the shortest gestational periods of all rodents (Graves, 1945). The mouse is born after 19 days of gestation. It has been reported that the hamster is born in a more immature state than other rodents (Schneider and Jhaveri, 1974), but studies of the general prenatal development of the hamster have found that the fetal hamster is one of the most rapidly developing mammals known (Graves, 1945; Ferm, 1967). In view of the hamster's immaturity at birth, it was expected that mitral and/or tufted cells would continue to divide in the early postnatal period. The results support, instead, the concept of a more compressed, rapid period of prenatal development. The olfactory bulbs of both hamster and mouse appear to be at a similar stage of histogenetic development at the time of birth.

In a preliminary analysis of the data, an attempt was made to classify the large cells in the external plexiform layer as either inner, middle, or outer tufted cells, as has been done previously (Ramon y Cajal, 1911; Hinds, 1968a). The initial analysis showed that the inner tufted cells were more closely related to the mitral cells in their time of origin than to the middle and outer tufted cells. The histological appearance in Nissl and Golgi stains of the inner tufted cells is very similar to the mitral cells. The depth analysis in this experiment demonstrates that there is also no clear histogenetic distinction between mitral and tufted cells, but rather a continuum of cells from the MCL through the EPL. The distinction between mitral and tufted cells was originally based on their positions in different layers of the bulb (Ramon y Cajal, 1911). Although it is now known that tufted cells project to olfactory cortical regions along with the mitral cells, rather than to the contralateral olfactory bulb, there are differences in the distribution of their projections. Evidence from HRP and electrophysiological studies indicates that tufted cells, especially middle and outer tufted cells, project more heavily to the olfactory tubercle than to piriform cortex (Haberly and Price, 1977; Scott et al., 1980, Scott, 1981). The relationship of these differential projections to the functional organization of the bulb or projection areas is yet to be determined.

One limitation to the present analysis is that ^{3}H-thymidine has only been injected at 24-hour intervals. The cell cycle of hamster cortical neurons has been estimated to be about 12 hours, with an S phase (the time of DNA synthesis when ^{3}H-thymidine is incorporated)

of about 6 hours (Shimada and Langman, 1970). It is not known if CNS cells cycle in synchrony. Injection of ^{3}H-thymidine at 24-hour intervals illuminates fairly narrow "windows" of cell formation, and labels only a fraction of the cells which actually go through their last S phase on that day.

Correlation of Birthdate and Axonal Projection

The second experiment demonstrated that in the hamster olfactory system, the axons of early-formed olfactory bulb cells reach the olfactory cortex before the axons of late-formed cells. The correlation of time of cell formation with axonal projections has been suggested in the rat retina, based on indirect evidence from diverse techniques. Cell formation in the retina generally proceeds from central to peripheral regions (Sidman, 1961), and occurs both pre- and postnatally. There is evidence from Golgi studies that axons from the central retina project into the optic tract when the peripheral retinal axons are still in the retinal optic nerve layer (Morest, 1970). Removal of one eye at birth produces an aberrant increased projection to the ipsilateral superior colliculus. Enucleation at 5 days of age results in an ipsilateral projection only to those areas innervated by the peripheral retina, again suggesting that the peripheral axonal projection develops later than the central projection (Lund et al., 1973). The present report is believed to be the first demonstration of the correlation of time of cell formation and axonal projection in individual cells.

The combination of the ^{3}H-thymidine and HRP labeling, each of which labels only a fraction of the possible cell population, results

in a fairly low expected proportion of double-labeled cells. In Experiment II the average predicted proportion of double-labeled cells was about 0.15%. There were about 350-400 mitral and tufted cells per section, which gives an expected frequency of approximately one double-labeled cell every two sections. Since about 200 sections were examined for each olfactory bulb in this experiment, the very low expected percentages translate into ample numbers of cells. The early-formed cells (E11) were double-labeled with a higher probability than predicted, while the late-formed cells were labeled considerably less than predicted.

Reinnervation after Transection of the LOT

After transection of the LOT at day 3, axons of cells formed on E11 are able to grow through the cut and reinnervate the olfactory cortex. Axons of cells formed on E13, on the other hand, rarely reinnervate the region caudal to the cut. Many of the E11 cells have axons which are already in the projection areas at the time of the transection, and would thus be cut by this procedure. Some axons of E11 cells may be well arborized along the course of the LOT, with established connections, while others may be just entering the region on their way to their final targets. Few axons of E13 cells have reached the level of the transection (which was generally at the level of the rostral olfactory tubercle) by day 3. Using autoradiographic methods to determine the LOT projection, Schwob and Price (1978) found that the LOT in the rat first innervates the region deep to the LOT, and then expands laterally, caudally, and medially. The data reported here suggest that the axons with the most extensive

projections (from E11 cells), rather than those that have not yet reached the region (from E13 cells), are the ones able to grow through the site of an early tract section. The projections rostral to the level of the cut (to the anterior olfactory nucleus and rostral olfactory tubercle) are probably not extensively arborized at 3 days of age, but may be sufficient to provide "sustaining collaterals" to support the cell. There may be other more mature features of the early-formed cells and their axons which help to maintain the cell and allow axonal regrowth. It is likely that the terminal fields are reinnervated by collateral sprouts from proximal branches of the transected fibers. The formation of collateral sprouts from both the proximal branches of the severed axons and nearby undamaged axons after lesions in young animals is now a well-established phenomenon (see, for example: Bjorklund et al., 1971; Lynch et al., 1973; Schneider, 1973; Pickel et al., 1974; Devor, 1976b; Kalil and Reh, 1979). Following an early LOT section, Devor (1976b) found both proximal collateral sprouting (rostral to the cut), and sprouting from the association system fibers caudal to the cut. It is possible that some of the proximal sprouts are able to continue through the cut into the more caudal projection regions. Axons of the later-formed cells (E13), which apparently cannot grow through the cut or subsequent scar may contribute to the increased density of innervation rostral to the cut, since these cells do not die. There was a loss of mitral cells following LOT section, apparently due to the loss of mitral cells formed on E12. These cells may have had their axons severed by the cut, but did not have sufficient proximal arborization (or maturity) to maintain the cell's viability.

Other Effects of Lateral Olfactory Tract Transection

When a tract section is done on day 3, some of the cells of origin in the bulb, especially tufted cells, have not yet completed their migration (as shown in Figures 6 and 7). At day 4, there are more cells in the mitral cell layer than in the EPL. As the perimeter of the MCL increases, the thickness of the MCL decreases. The reversal in the ratio of mitral to tufted cells, such that at one month of age there are more tufted than mitral cells, indicates that many cells in the MCL at day 4 are actually passing through the MCL and will eventually be located in the EPL. Figure 7 shows additionally that the number of cells in the MCL at day 4 is greater in animals injected with ^{3}H-thymidine on E13 than on E11. Transection of the LOT at day 3 results in a change of position within the EPL of cells labeled with ^{3}H-thymidine on E13. These cells have not moved as far into the EPL as they normally would have (Figure 8). In addition, the number of total (labeled and unlabeled) tufted cells is increased after LOT section in animals given ^{3}H-thymidine on E13, but not those injected on E11. This suggests that the altered position at the time of tract section of a cell destined for the EPL affects the final position the cell achieves. A possible mechanism for this effect is proposed in the following section.

Aberrations in Developmental Interactions as Possible Consequences of Mild ^{3}H-thymidine Toxicity

In preliminary studies, animals were given 5μCi/gm of ^{3}H-thymidine on various days of gestation and in the early postnatal period. This dose is higher than that required to label cells in other organ

systems, but it is a common dose for studies of the CNS (Sidman, 1970). Although the hamster has not been found to have any deficiencies in thymidine incorporation (Adelstein et al., 1964; Adelstein and Lyman, 1968), the dose of 5μCi/gm resulted in extremely light labeling of cells, even after long exposure times (up to 13 weeks). The labeling was especially light in animals injected early in the gestational period (E10-11). The embryonic circulation is still developing at this time, and it is likely that less thymidine is available to the embryo for incorporation than later in development, given equivalent intraperitoneal injections to the mother (Atlas et al., 1960; Taber Pierce, 1967; Boyer, 1968). We were fairly confident that some cells in the population examined ought to be undergoing their last divisions and thus be heavily labeled, so the dose of ^{3}H-thymidine was increased to up to 10μCi/gm, given either in a single injection or in two injections, one hour apart. Since intraperitoneally injected ^{3}H-thymidine is available for nuclear incorporation for about one-half to one hour (Sidman, 1970), giving two injections (in the context of an S phase of about 6 hours) effectively increases the exposure time for incorporation of thymidine. Lower doses of ^{3}H-thymidine result in more effective uptake and retention of the thymidine, so that incorporation after two smaller doses is greater than if the same total amount was given in one dose (Samuels and Kisieleski, 1963). The higher dose produced identifiable labeling of all cell types.

As in many other studies of CNS histogenesis (for example: Hinds, 1968a; Altman, 1969; Fujita, 1967), there was no increase in the incidence of abortion or maternal or pup mortality, and no gross developmental defects were observed. The results of this experiment,

however, suggest that the injection of ^{3}H-thymidine on E13 has an effect on the position of cells destined for the EPL that incorporate the ^{3}H-thymidine at this time. Four pieces of evidence led to this conclusion. 1) At 4 days of age, cells labeled with ^{3}H-thymidine on E13 have not yet reached their final positions in the EPL (as shown in Figure 6). This is likely to partially reflect their normal course of migration, but 2) the number of total cells--labeled and unlabeled--in the MCL at day 4 is greater after ^{3}H-thymidine injection on E13 than on E11 (Figure 7). The normal course of migration of unlabeled cells would not be expected to be different in these two groups. Injection of ^{3}H-thymidine on E13 appears to delay or prolong the migration of cells in which it is incorporated. 3) Tract section on day 3 also affects the positions of E13 ^{3}H-thymidine-labeled cells (Figure 8). 4) Tract section on day 3 causes an increase in the number of tufted cells, many of which are formed on or after E13 (Figure 9). These results have led to the speculation that perhaps the injection of ^{3}H-thymidine on E13 has a mildly toxic effect on cells that undergo their last division on or after that time. This effect is expressed as delayed migration or a decreased rate of migration, although the initial effect could be to lengthen the cell cycle or interfere with mitosis.

Cytological damage, cell death, and tumor induction have been reported after doses of ^{3}H-thymidine as low as 1-10μCi/gm (Baserga et al., 1962; Samuels and Kisieleski, 1963; Kisieleski et al., 1964). The biological effect of ^{3}H-thymidine is related to the dose of ^{3}H-thymidine incorporated into the nucleus, which depends upon the dose administered, the specific activity, and the age of the animal

(Baserga et al., 1962; Samuels and Kisieleski, 1963; Bond and Feinendegen, 1966; Blenkinsopp, 1967). The doses of 5-10μCi/gm commonly used in studies of CNS histogenesis are well within the range known to cause damage, but effects less severe than cell death may not be readily detected. Olsson (1976) has shown that ^{3}H-thymidine (1μCi/gm) produces a temporary inhibition of DNA synthesis and a delay in mitotic activity of labeled cells in mouse epidermis. Mitotic activity of unlabeled cells was increased by ^{3}H-thymidine injection. Hicks and d'Amato (1968) found a decreased number of neurons in layers II and III of rat cortex after injection of 16.4μCi/gm on E17 (22 day gestation). They were not able to determine if this was due to a delayed migration or cell death. Because of its lamination and distinct developmental pattern, the olfactory bulb provided an ideal system where it was possible to count both labeled and unlabeled cells, and to follow the relative positions of cells with known times of origin without the presence of labeled thymidine in the cells. The toxic effect was only detectable by the comparison of injections on E11 and E13. Although there may have been an effect of E11 injection, the relatively greater effect was seen in the E13-injected animals. It is likely that the effective incorporated dose of ^{3}H-thymidine was less in the E11 animals due to a relative immaturity of the circulation. Even with higher doses of ^{3}H-thymidine, cells were more lightly labeled after E11 injections, which also suggests that less ^{3}H-thymidine was incorporated.

A delayed migration or decreased rate of migration of E13 cells could explain both the increase in total tufted cells and the decreased $\bar{r}$ of thymidine-labeled cells after LOT section in E13 animals. At 3

days of age, cells formed on E13, in their course of migration, are likely to be passing by earlier-formed cells in the MCL and lower EPL. Under normal circumstances, they would pass by E12 cells and proceed to the appropriate level in the EPL. Cells formed on E14 would also proceed in this manner, some of which may continue up into the glomerular layer, where they are not easily identifiable as tufted cells. In E11 or uninjected animals, following LOT section on day 3, some E12 cells die, accounting for the decreased number of mitral cells, but the E13 and E14 cells are already passing the E12 cells and continue to their normal positions. When the animal has received ^{3}H-thymidine on E13, the E13 and E14 cells arrive at the MCL later, and may not have reached the E12 cells by the time the E12 cells die. If one of the cues for termination of migration comes from the positions of other cells which are being passed along the course of migration, the E13 and E14 cells might assume lower positions in the EPL when the population of E12 cells is reduced. The presence of some E14 cells in the EPL which otherwise would have passed up into the glomerular layer could result in the increased number of tufted cells in this case.

Both an increase in the duration of the cell cycle (Kauffmann, 1968; Hoshino et al., 1973) and a decrease in the rate of migration (Hicks and d'Amato, 1968; Hinds, 1968b) are found to occur during normal embryonic development. The differences in the positions of the total cells between animals injected on E11 and E13 indicate that there is an additional effect of ^{3}H-thymidine injection on E13. These results suggest that studies of time of cell formation and migration should be thoughtfully interpreted, since the measuring instrument (^{3}H-thymidine) may influence the phenomenon being measured (Olsson, 1976).

Possible Mechanisms of Reinnervation after Early Lesions

We have found that the axons of early-formed cells, which have reached the olfactory projection regions early, are able to reinnervate these regions after tract section on day 3. One question which has not been answered by these experiments is why the LOT axons are not able to regrow and reinnervate the projection regions when the tract is cut after day 7. The results of these experiments show that it is not due to the normal termination of new axons growing into the area at this time. Other experiments have shown that while the capacity for regrowth progressively decreases to day 7 (Devor, 1976b), there is a large increase in the innervation of the olfactory cortex after day 7 (Grafe and Leonard, 1981). From the experiments reported here, it appears that the proximal parts of the more mature axons are better able to survive the transection and then move through the cut or scar. Mature axons do retain some capacity for collateral sprouting (Lynch et al., 1973) and Barker and Ip (1966, p. 550) found that "motor axons of mammals undergo collateral and ultraterminal sprouting under normal conditions" as well as when deafferented. The critical changes in development which prevent axonal regrowth after day 7 appear to be not in the cells of origin and their axons, but rather in the tissue into which the axons are growing.

The end of the first postnatal week is approximately the time when long-lasting degeneration argyrophilia appears in the olfactory projections (Leonard, 1975). The persistence of degenerating fibers could influence the ability of axons to reinnervate the area (Westrum, 1980). During this time there is also an increase in the growth of the olfactory assocaition projections (Price et al., 1976; Singh, 1977;

Schoenfeld et al., 1981). Regrowing axons may not be able to compete as well for synaptic space with association axons which had never been interrupted. The glial and connective tissue scar which forms after CNS injury has been recognized for many years to interact with axonal regenerative attempts (Windle, 1956). The scar may serve not only as a barrier (Windle, 1956; Puchala and Windle, 1977), but may also actively deviate growing axons. Regenerating axons have been found to associate with loose connective tissue and be deflected by dense connective tissue (Fertig et al., 1971). In the developing and regenerating newt spinal cord, axons are guided by channels formed by the ependymal cells and their processes (Singer et al., 1979). The scar is not an absolute barrier, however, since regenerating adrenergic axons can grow through the scar (Bjorklund et al., 1971; Nygren et al., 1971). The early descriptions of regenerative attempts by Ramon y Cajal (1928) report that axons can grow into the scar, but then stop their growth. Windle (1956) and Puchala and Windle (1977) report pharmacological manipulations which allow axons to grow into the region of the scar, but these also do not survive. There may be developmental changes in the density or composition of the scar, or in the presence and form of phagocytic cells in the region of the scar, but the effects these factors may have on axonal regeneration are not clear.

If the potential for reinnervation had been shown to be limited by the period of axonal growth, the possibility for functional recovery would be quite limited. The results of this study thus add a positive note to the current views of the restorative capacities

of the CNS. The difficult questions of what the critical developmental factors influencing axonal regrowth are, and whether they can be modified in a functionally beneficial manner remain to be investigated.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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